Project ID 367

Competitive Research Grant

Sub-Project Completion Report

on

Development of Live Attenuated Duck Plague Vaccine Using Local Duck Plague Virus Strains

Project Duration

July 2017 to September 2018

Department of Microbiology & Hygiene, Bangladesh Agricultural University, Mymensingh

Submitted to

Project Implementation Unit-BARC, NATP 2
Bangladesh Agricultural Research Council
Farmgate, Dhaka-1215

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Project Implementation Unit
National Agricultural Technology Program-Phase II Project (NATP-2)
Bangladesh Agricultural Research Council (BARC)
New Airport Road, Farmgate, Dhaka – 1215
Bangladesh

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Acknowledgement

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Acronyms

%	:	Percentage
µl	:	Microliter
BAU	:	Bangladesh Agricultural University
bp	:	Base pair
C	:	Degree celcius
CAM	:	Chorioallantoic membrane
Co-PI	:	Co Principal Investigator
DLS	:	Department of Livestock Service
DNA	:	Deoxyribo nucleic acid
DP	:	Duck plague
DPV	:	Duck plague virus
F	:	Forward
Fig.	:	Figure
hrs	:	Hours
LRI	:	Livestock Research Institute
MS	:	Masters of Science
PCR	:	Polymerase chain reaction
PI	:	Principal Investigator
R	:	Reverse
rpm	:	Rotation per minute
TAE	:	Tris Acetate EDTA
USA	:	United States of America
UV	:	Ultraviolet
Yr	:	Year

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Executive Summary

Duck plague (DP) is one of the devastating diseases of duck. It is caused by a DNA virus belongs to the family herpesviridae. It causes enormous economic losses in the duck farms. In order to protect the ducks from this disease an effective vaccine using field isolates need to be developed. The goals of this research project were isolation and characterization of duck plague virus from natural outbreaks and preparation of a live attenuated DP vaccine using the local strain.

A total of 18 dead ducks and swans were collected from Mohongonj, Netrokona (n=4), Tarati, Mymensingh Sadar Upazilla (n=6), Nilphamari, (n=4) and Safari Park (n=4), Gazipur from February to June 2018. The flock size of the affected farm ranged between 20 and 2000. The age of the affected ducks ranged from 3 weeks to >1 year. Liver, spleen, kidney and lungs tissues were collected aseptically from the dead duck during post-mortem examination.

A 10% tissue homogenate was prepared from the liver and spleen. The DNA was extracted from the tissue homogenate. A PCR assay targeting 446 bp DNA fragment of DNA polymerase gene was carried out for identification of duck plague virus (DPV) in the liver and spleen tissues. Tissue suspension was treated with antibiotic (Gentamicin) and antifungal (Nystatin) agents to prepare viral inoculum. Processed tissue homogenate (inoculum) was inoculated into 12 day old embryonated duck eggs through chorioallantoic membrane (CAM) route and incubated at 37°C for 6 days for isolation of DPV.

The CAM was harvested from the embryonated duck and 10% tissue homogenate was prepared. The viral DNA was extracted from the CAM homogenate and PCR was performed to confirm DPV. Molecular characterization of duck plague virus was performed by sequence analysis of the 446bp fragment of DNA polymerase gene and phylogenetic tree was constructed to know the origin of the virus.

The ducks in the affected farms manifested the major clinical sings of anorexia, ocular and nasal discharge, ataxia and death. Enlarged and hemorrhagic spleen and liver were seen in the affected ducks during post-mortem examination. Congestion and hemorrhage were observed in the intestine. Annular hemorrhagic rings were seen in the trachea of the affected ducks. The characteristic clinical signs and post mortem lesions indicated that the ducks were infected with duck plague virus. The morbidity rate of duck was ranged from 35-60% with the mortality rate of 15-46.7%. Degeneration of blood vessels in the CAMs including death and dwarfism of the embryos were noticed following experimental inoculation of embryonated duck eggs with DPV.

The DPV were identified in the field samples as well as in the CAM of the experimentally infected embryos using PCR as evidenced by the production of 446bp PCR amplicons of DNA polymerase gene. The phylogenetic analysis of the DPV isolates under this study showed 100% sequence similarity with DPV isolates originated from Bangladesh, Vietnam and China. For attenuation of the virulent DPV, 0.1 ml of the virus isolates was inoculated into 9- 12 day old embryonated chicken eggs and incubated at 37°C for 3-4 days. Out of the total 46 passages required for attenuation of virulent DPV only 10 passages were completed during the project period. The virus in the tissue homogenate from each of the passages was confirmed by PCR assay. Due to time constraints the objectives of the development of live attenuated duck plague vaccine and determination of its efficacy could not be completed.

CRG Sub-Project Completion Report (PCR)

A. Sub-project Description

1. **Title of the CRG sub-project:**
   Development of Live Attenuated Duck Plague Vaccine Using Local Duck Plague Virus Strains

2. **organization:**
   Bangladesh Agricultural University, Mymensingh

3. **Name and full address with phone, cell and E-mail of PI/Co-PI (s):**
   Dr. Md. Ariful Islam
   Professor, Department of Microbiology & Hygiene
   Bangladesh Agricultural University
   Mymensingh-2202
   Phone: 01711390939, e-mail: islamma@bau.edu.bd

4. **Sub-project budget (Tk):**
   4.1 Tk. Total: 24,00,000.00
   4.2 Revised (if any):

5. **Duration of the sub-project:**
   5.1 Start date (based on LoA signed) : 12 July 2017
   5.2 End date : 30 September 2018

6. **Justification of undertaking the sub-project:**

Duck is the second largest poultry in Bangladesh after chicken. Bangladesh has ranked 3rd in terms of number of duck population (38.1 millions) in the world (Dolberg, 2008). Duck is reared throughout Bangladesh. However, number of duck farms is more in the Haor areas of Bangladesh. Duck plays important role in poverty reduction of rural peoples and contributes to food security as well as human nutrition since its meat and egg are very good sources of protein. Ducks are resistant to many infectious diseases and feeding costs is less compared to chicken which made duck farming a profitable enterprise in Bangladesh. Duck farming has the huge potentiality for income generation of the rural woman and unemployed youth. Although, ducks are resistant to many diseases but they are very sensitive to duck plague. It is a very fatal viral disease of duck caused by a DNA virus that belongs to the sub family alpha herpesvirinae under the family herpesviridae. Duck plague causes 60-70% mortality in duck In Bangladesh (Sarker, 1982). This disease is considered as a great obstacle to duck farming in Bangladesh (Hoque *et al*., 2010). Imported and locally produced vaccines are used for prevention of this deadly disease. Kayesh et al. (2008) conducted a study on the standardization of effective vaccine dose and best age of vaccination for duck plague vaccine produced from the Livestock Research Institute (LRI), Mohakhali, Dhaka. The efficacy of duck plague vaccine produced by LRI has been reported to produce good immunity against DP infection (Hossain *et al.*, 2005). However, nowaday vaccines produced by LRI are always inadequate against its current demand. In addition, the quality of the vaccine is not always satisfactory (Personal communication) and sometimes it fails to protect the ducks despite regular vaccination (Kayesh et al., 2008). Despite vaccination duck plague outbreaks are often reported, which raise the question of efficacy of currently available vaccine in Bangladesh. It is generally accepted that if vaccine is produced from local strain of virus often confer

good protective immunity against a specific viral disease. In order to protect the duck from duck plague there is an urgent need to develop an effective vaccine against duck plague using local strain of viruses isolated from natural outbreaks.

7. Sub-project goal:
Development of a live attenuated duck plague vaccine to protect the ducks from duck plague

8. Sub-project objective (s):
a) Isolation, identification and characterization of duck plague virus (DPV) from field outbreaks
b) Development of live attenuated duck plague vaccine from local field strains
c) Determination of efficacy of the developed vaccine

9. Implementing location (s):
Department of Microbiology & Hygiene, Bangladesh Agricultural University, Mymensingh-2202

10. Methodology in brief:

10.1. Isolation, identification and characterization of duck plague virus (DPV) from field outbreaks

Approach
Contact (by personal visit and mobile phone) was established with the duck farmers, Upazilla livestock officer, Veterinary surgeons and livestock health workers at Netrokona, Sunamgonj, Mymensingh and Nilphamari districts to indentify duck plague outbreak farms and flocks. On report of the duck plague outbreaks, field sample collection team (comprised of MS fellow and /or PI) went to outbreak spots and collected samples from sick and dead ducks for lab analysis.

Collection of Sick and dead duck samples
Samples were collected from ducks during natural outbreaks (February to June 2018) of duck plague from four districts of Bangladesh (Table 1).

Table 1. Detail information of sample collection from ducks during natural outbreaks of duck plague

Study area	Age of ducks	Flock size	Breed of ducks	No. of samples collected	Vaccinated with DPV or not
Mohanganj, Netrakona	6 months - 1 yr	800	Khakhi Campbell	4	Not vaccinated
Tarati, Mymensingh	3 weeks	2000	Khakhi Campbell	6	Not vaccinated
Kishoregonj Nilphamari,	3 weeks	1500	Khakhi Campbell	4	Not vaccinated
Safari park, Gazipur	Age not known	20	Australian black swan	4	Vaccinated

Post-mortem examination and collection of samples

A through post-mortem examination was carried out (Figure 1). Samples were collected aseptically during post-mortem examination of dead ducks. Visceral organs such as liver, spleen, kidney, trachea and intestine were collected and kept separately in sterile falcon tubes and stored at -20^{0}C until use.

Figure 1: Performing post-mortem examination of a duck suspected of DPV infection

Preparation of inoculum

Samples (liver and spleen) were cut into small pieces using sterile scissor and forceps and grinded using sterile pestle and mortar (Figure 2). Then sufficient amount of PBS was added to make 10% tissue suspension. Samples were then centrifuged at 6000 rpm for 20 minutes. The supernatant fluid was collected and treated with antibiotic (Gentamycin) and antifungal (Nystatin) agents. Sterility of inoculum was performed by culturing onto blood agar at 37^{0}C for 24 hours. Bacteriologically sterile inoculum was stored at -20°C until use.

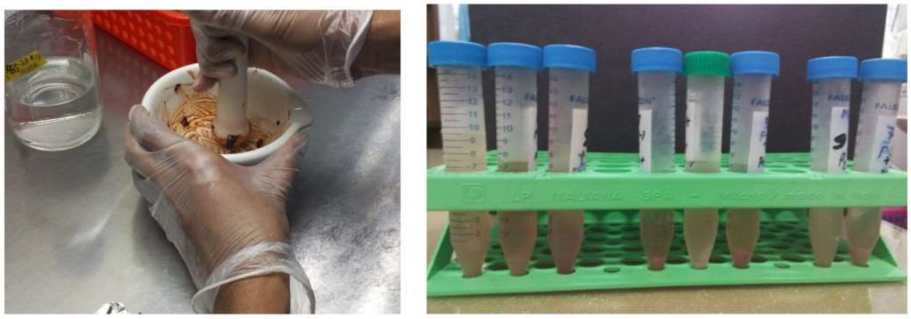

Figure 2: Grinding of liver and spleen (left) to prepare 10% tissue homogenate (right)

Extraction of viral DNA from tissue suspension

DNA was extracted from 10% tissue homogenates of spleen and liver of ducks suspected to be infected with DPV using a commercially available Kit (Gene Proof, Brno, Czech Republic*)* according to the instruction of the manufacturer (Figure 3).

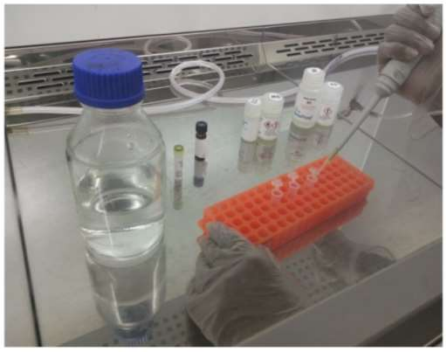

Figure 3: Activities showing DNA extraction from clinical specimens

Molecular detection of duck plague virus in the field samples by PCR

The DNA extracted from the field sample was used as template for PCR based detection of DPV in the samples. The PCR targeting 446 bp DNA fragment of DNA polymerase gene of duck plague was performed according to Wu *et al*. (2012). The oligonucleotide sequences used in primers for PCR and thermal profile of PCR reaction are listed in Table 2 and Table 3 respectively.

Table 2: Oligonucleotide sequences used for PCR

Name of genes	**Primers**	**5'-Sequence-3'**	**Amplicon size**
DNA polymerase	F	5´-GAAGGCGGGTATGTAATGTA-3´	446-bp
	R	5´-CAAGGCTCTATTCGGTAATG-3´	

Table 3: PCR reaction profile for amplification of DNA polymerase gene of DPV

Steps	Temperature(°C)	Time(min)	Cycle
Initial denaturation	94	2	1
Denaturation	94	1	35
Annealing	56	1	
Extension	72	2	
Final extension	72	7	1
Hold	4	∞	

Isolation of virus using embryonated duck egg

Preparation of embryonated duck eggs

Duck eggs were purchased from a duck breeding farm. The surface of the egg was cleaned by 70% alcohol. The cleaned eggs were kept in an incubator set at 37^0C temperature with relative humidity of 85% for 9-12 days (Figure 4). Eggs containing well developed healthy embryos were selected for inoculation.

Figure 4: Cleaning of eggs with 70% alcohol (left) and placing eggs in an egg incubator (right)

Inoculation of viral inoculum into embryonated duck eggs

Eggs containing well developed healthy embryos of 12 days old were selected for inoculation. The inoculation sites on eggs were marked with pencil avoiding blood vessels. The sites were disinfected with tincture of Iodine. Egg driller was used to make holes on marked points of the eggs. An artificial air sac was created over the CAM by sucking air using a rubber bulb. Bacteria free viral inoculum of 0.2 ml volume was inoculated into each embryonated egg through CAM route using a sterile tuberculin syringe fitted with 26-27 gauze needle (Figure 5). The holes were sealed off with liquid paraffin or wax after inoculation. Inoculated eggs were placed in an egg incubator at 37^0C for 05 days. During incubation the eggs were observed twice daily for mortality of the embryos (Figure 5). The embryos died within 24 hours of inoculation were discarded. The embryos died after 24 hours were collected and kept in a refrigerator at 4°C until use.

Figure 5: Inoculation (left) and candling of eggs for detection of viability of inoculated embryos (right)

Identification of virus

Harvesting of CAM and preparation of embryo homogenate

The embryos together with the CAMs were harvested aseptically into a petri dish and the lesions were recorded (Figure 6). The heads and limbs of the embryos were discarded. The main body of the embryos was homogenized together with the CAM in PBS to make a 10% embryo suspension.

Figure 6: Collection (left) and homogenization of CAM and body of embryo (right)

Extraction of viral DNA from CAM and embryo homogenate

The DNA from CAM and embryo homogenate was extracted by commercially available viral DNA extraction kit (GeneProof, Brno, Czech Republic*)* using manufacturer's protocol. The DNA was used as template for PCR.

Molecular detection of duck plague virus by PCR

PCR assay was performed to identify DPV by amplifying 446 bp DNA polymerase according to the protocol described by Wu *et al*. (2012).

Gel electrophoresis of PCR products

The procedure used in the gel electrophoresis is listed below:

- **i.** Gel casting tray was assembled with gel comb of appropriate teeth size and number.
- **ii.** 1.5% agarose solution was prepared in TAE buffer by melting in a microwave oven.
- **iii.** Molten agarose was poured onto the casting tray and allowed to solidify on the bench.
- **iv.** The solid gel in its tray was transferred to the electrophoresis tank containing sufficient TAE buffer to cover the gel ≈1mm. The comb was gently removed.
- **v.** 5 µl of each PCR product was mixed with 1µl loading buffer and the sample was loaded to the appropriate well of the gel.
- **vi.** 5 µl DNA size marker (Trackit, invitrogen, USA) was loaded in one well.
- **vii.** The leads of the electrophoresis apparatus were connected to the power supply and the electrophoresis was run at 80 V for 28 minutes.
- **viii.** When DNA migrated sufficiently, as judged from the migration of bromophenol blue of loading buffer, the power supply was switched off.
- **ix.** The gel stained in ethidium bromide (0.5µg/ml) for 10 minutes, in a dark place.
- **x.** The gel was de-stained in distilled water for 10 minutes. The de-stained gel was placed on the UVsoloTS imaging system (Biometra, Germany) in the dark chamber of the image documentation system.
- **xi.** The UV light of the system was switched on, the image was viewed on the monitor, focused, acquired and saved in an USB flash drive.

Molecular characterization of DPV by Sequence analysis and construction of a phylogenetic tree

The PCR product of partial amplified DNA polymerase gene (446 bp) was sequenced from a commercial company (Apical Scientific Sdn Bhd, Selangor, Malaysia). The nucleic acid sequence obtained from the PCR products was aligned with other 24 known sequence of DPV available in the GenBank, and a phylogenetic tree and divergence table were constructed based on neighbor joining method (Saito and Nei , 1987) using MEGA 6 (Tamura et al., 2004) software.

Analysis of evolutionary divergence between sequences was conducted using the Maximum Composite Likelihood model (Tamura *et al.,* 2004). The analysis involved 27 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 71 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6 (Tamura *et al.*, 2013).

10.2. Development of live attenuated duck plague vaccine from local field strains:

Virus attenuation

Virulent DPV inoculum (0.1ml) was inoculated into 9 -12 day old embryonated chicken eggs and incubated at 37^{o}C for 3-4 days. CAM and embryos of the chicken embryos were collected and 10% tissue

homogenate was prepared as described earlier. The DPV in the tissue homogenate was confirmed by PCR. The process of embryo inoculation and PCR assay was repeated for each passage throughout the attenuation process of live virus.

10.3. Determination of efficacy of the developed vaccine

The activities for the development of live attenuated DPV vaccine and the determination of efficacy of the developed vaccine could not be started due time constraints.

11. Results and discussion

11. 1. Isolation, identification and characterization of DPV from field outbreaks

Duck plague outbreaks in the study areas

Outbreaks of duck plague (DP) was found to occur in the farms located at Mohangonj, Netrokona, Mymensingh Sadar, Nilphamari and in the Safari park, Gazipur during February to June of the year 2018. The morbidity rate of duck ranged between 35 to 60% and mortality rate was between 15 to 46.7%. The major clinical signs manifested by the affected ducks were anorexia, ocular and nasal discharge, ataxia and death. The detailed information of the outbreak, morbidity, mortality and clinical signs of the affected duck is presented in Table 4.

Table 4: Detail information of duck plague outbreaks and clinical signs manifested by the affected ducks

Study area	Flock size	No. of affected (Morbidity)	No. of dead (Mortality)	Clinical sign manifested by ducks	Interpretation
Mohanganj, Netrakona	800	350 (43.75%)	200 (25%)	• Depression • Drop in egg production • Ruffled and dull feathers • Ocular and nasal discharge • Anorexia, labored breathing • Watery diarrhea • Extreme thirst • Ataxia and death	**Duck plague**
Tarati, Mymensingh	2000	700 (35%)	300 (15%)		
Kishoregonj, Nilphamari	1500	900 (60%)	700 (46.7%)		
Safari park, Gazipur	20	10 (50%)	6 (30%)		

The rate of morbidity and mortality due to DP varies from 5-100% (Calnek *et al.,* 1997). Huque and Husain (1994) reported 58% mortality in Khaki Campbell and 72% mortality in deshi duck. A study conducted at Netrokona recorded 27.1% mortality of duck due to DPV infection (Khanum *et al.,* 2005). Islam *et al.* (2003) and Sarker (2005) reported 6-9% mortality in indigenous growing duck due to DPV. The duck plague occurs in 7 days old duckling to mature ducks (Walker *et al.,* 1969). In the present study the age of the affected ducks was between 3 weeks to one year. The mortality rate of duck plague is usually higher in adult breeder ducks (Proctor, 1975). The spontaneous shedding of virus reported from convalescent duck during spring season (Dhama *et al.,* 2017). In this study the outbreaks of duck plague occurred mostly from March to June.

Post-mortem findings

A detailed post-mortem examination was carried out on the dead ducks collected from the duck plague affected farms. The gross lesions of the spleen, liver, trachea and abdominal cavity were recorded during post-mortem examination (Table 5). Similar post-mortem lesions were also seen by several investigators (Shawky *et al.,* 2000; Campagnolo *et al.,* 2001). Dhama *et al.* (2017) and Hansen *et al.* (2007) stated that viral replication causes an increase in vascular permeability, which leads to the development of lesions and hemorrhages in the liver and spleen. The gross lesion observed in various organs (Figures 7-10) indicated that the ducks on the affected farms were infected with duck plague virus.

Table-5. Post-mortem findings of spleen, liver, trachea and abdominal cavity of the DP affected ducks

Organ examined	Gross lesions observed	Interpretation
Spleen	Large and hemorrhagic spleen of a duck (Fig. 7)	Duck plague virus infection
Liver	Large and hemorrhagic liver of a duck (Fig. 8)	
Trachea	Annular hemorrhagic ring on the trachea of a duck (Fig. 9)	
Abdominal cavity	Hemorrhage in the body cavity of a duck (Fig. 10)	

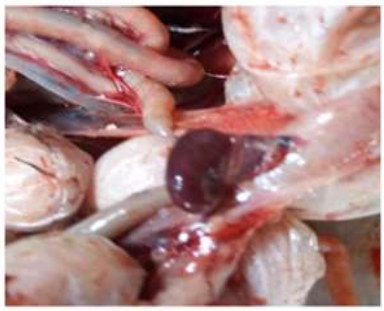

Fig. 7: Large and hemorrhagic spleen of a duck

Fig. 8: Large and hemorrhagic liver of a duck

Fig. 9: Annular hemorrhagic ring on the trachea of a duck

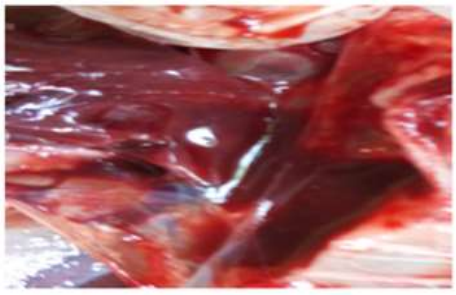

Fig. 10. Hemorrhage in the body cavity of a duck

Molecular detection of duck plague virus in the field samples

The PCR based detection methods are sensitive and accurate for detection of duck plague virus (Dlugosch *et al*., 1991; Williams *et al.,* 1992). The DNA polymerase genes of duck plague virus are important target for molecular detection of DPV.

A total of 18 samples obtained from duck plague affected farms were subjected to PCR based detection of DPV. The PCR was performed using DNA extracted from field samples (spleen and liver) targeting 446 bp DNA fragment of DNA polymerase gene of DPV. The results of PCR assay are furnished in Table 6 and Figures 11-14.

Table 6: Results of molecular detection of duck plague virus (DPV) by PCR assay

Duck plague outbreak area	No. of samples screened by PCR	No. of DPV positive samples (%)
Mohanganj, Netrakona	**4**	**4 (100)**
Tarati, Mymensingh	**6**	**1 (16.66)**
Kishoregonj, Nilphamari	**4**	**3 (75)**
Safari park, Gazipur	**4**	**2 (50)**

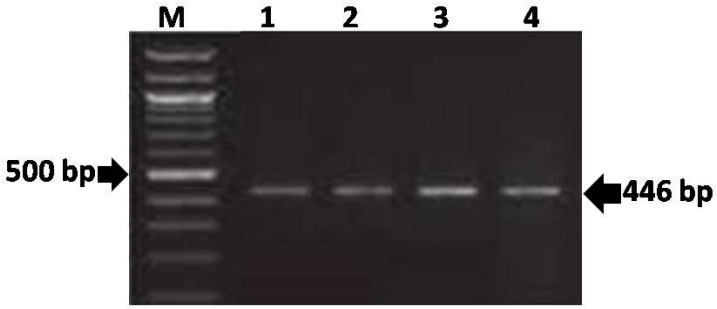

Figure 11: PCR amplification of DPV DNA from the duck samples of Mohangonj, Netrakona
Lane M: 100 bp size DNA ladder, lanes 1-4: DNA from liver and spleen samples of ducks.

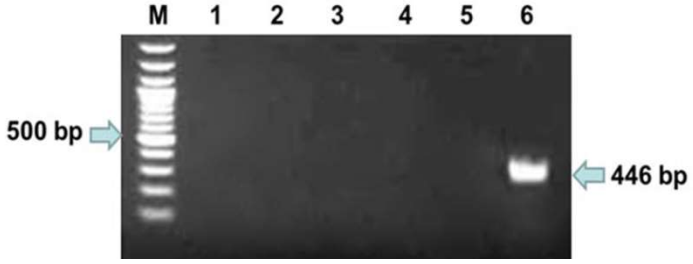

Figure 12: PCR amplification of DPV DNA from the duck samples of Tarati, Mymensingh
Lane M: 100 bp size DNA ladders, lanes 1-6: DNA from liver and spleen samples of ducks.

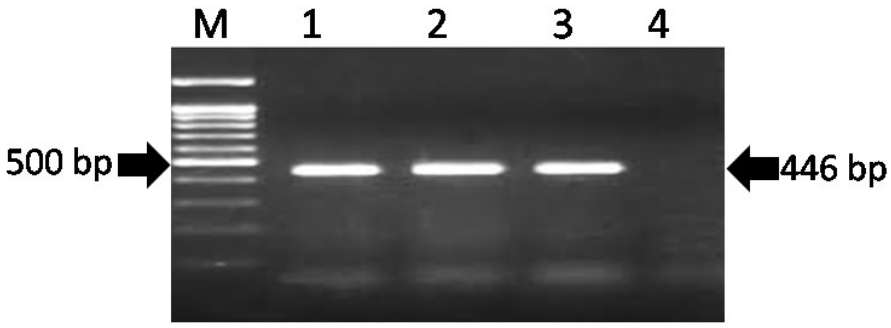

Figure 13: PCR amplification of DPV DNA from the duck samples of Kishoregonj and Nilphamari
Lane M: 100 bp size DNA ladders, lanes 1-4: DNA from liver and spleen samples of ducks

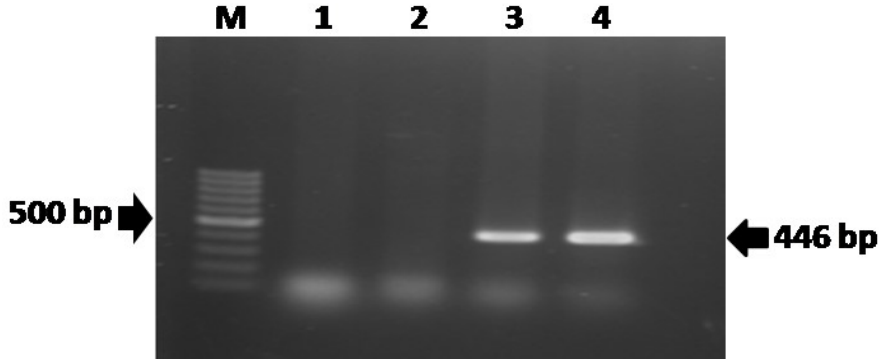

Figure 14: PCR amplification of DPV DNA from the duck samples of the Safari park, Gazipur
Lane M: 100 bp size DNA ladders, lanes 1-4: DNA from liver and spleen samples of Australian black swans

11.2. Development of live attenuated duck plague vaccine from local field strains:

Virus attenuation

The duck plague virus positive inoculum was inoculated into 12 day old embryonated duck for propagation of virus. Embryo died before 24 hrs of inculcation was considered as non-specific death and did not examine for gross lesion. The embryo died after 24 hrs was examined for gross lesions. Degeneration of blood vessels CAM of infected duck was observed to compare with the uninfected control CAM and dwarfism was also observed in the infected embryos when compared with the infected control embryo (Figure 15). In the present study the duck embryos died between 2 and 5 days following experimental infection. Degeneration of blood vessel of CAM and dwarfism of embryos were seen. Similar findings were also observed by Jansen *et al.* (1968), Akter *et al.* (2004).

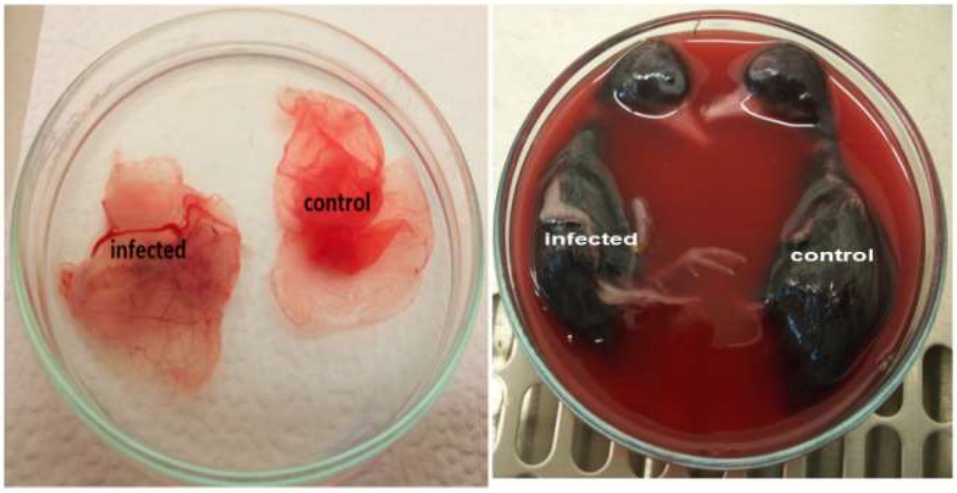

Figure 15: Duck plague virus infected CAM (left) and embryo (right) with the uninfected controls
Degeneration of blood vessel is seen in the duck plague virus infected CAM and dwarfism of duck embryo is seen in the experimentally infected embryo as compared to uninfected controls

Detection of Duck plague virus in embryonated duck eggs

In this study DPV was propagated in the embryonated duck eggs. Jansen *et al.* (1968) obtain highest virus titer using CAM route for the propagation of DP virus. A total of 10 duck plague virus positive inoculums were inoculated separately into 12 day old duck embryos. The CAMs were harvested and 10% tissue homogenate was prepared. The DNA was extracted from tissue homogenate and PCR assay was performed which successfully detected 08 isolates of DPV with amplification of 446 bp PCR amplicons. The results of PCR assay using DNA of CAM of duck embryos are presented in Table 7 and Figure 16.

Table 7: Results of amplification of duck plague virus DNA from CAM of embryonated duck using PCR

No. of CAM homogenate tested	No. of PCR positive embryos (%)	No. of PCR negative embryo (%)
10	08 (80)	02 (20)

CAM: Chorio-allantoic membrane

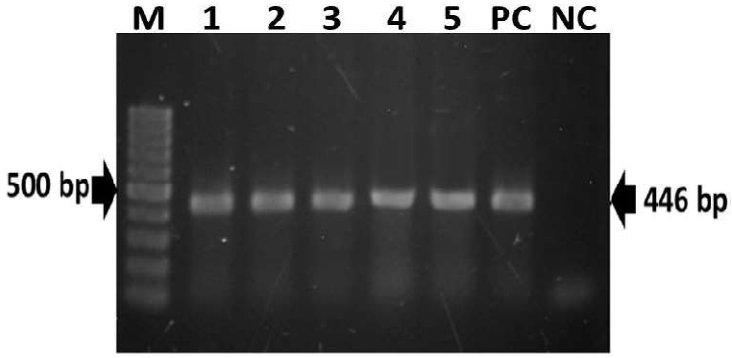

Figure 16: PCR amplification of DPV DNA from CAM homogenate of embryonated duck
Lane M: 100bp size DNA ladder, Lanes 1-5: DNA from CAM homogenate of embryonated duck, Lane PC: positive control, Lane NC: negative control.

Molecular characterization of the local isolates of duck plague virus

Sequencing and Phylogenetic analysis

The partial sequences (446 bp) of DNA polymerase gene of three DPVs are as follows:

*5'*GAAGGCGGGTATGTAATGTACATTCCATTTACTGGAAATGCCGTACATCTACACTATCGT
CTCATCGACTGCCTTAAATCTGCTTGCCGGGGATACCGTCTAATGGCTCATGTTTGGCAT
TCTACATTCGTACTTGTCGTGAGGCGCGACCGCGAACGGCAAACTGACGTGGACAGCGTA
CCACAGATAAGTATTGAAGATATTTATTGTAAAATGTGCGACCTTAATTTCGATGGGGAA
CTTCTGCTAGAATATCGAAAGCTCTACGCAGCTTTTGACGATTTTCCTCCTCCTCGCTGA
GTGGCATCCCTGGGTACAAGCGCACTTCTGCAAACCCGGCCGAAGATAGCAGTGCTGCGG
TTTCGTCACTCTCACAGTATGTTTCTGGAATAAAGCGTTTTAAAACAGCTTCCGAAGTTT
TGTGATCATTACCGAATAGAGCCTTG *3'* (Isolate no. 1)

*5'*GAAGGCGGGTATGTAATGTACATTCCATTTACTGGAAATGCCGTACATCTACACTATCGT
CTCATCGACTGCCTTAAATCTGCTTGCCGGGGATACCGTCTAATGGCTCATGTTTGGCAT
TCTACATTCGTACTTGTCGTGAGGCGCGACCGCGAACGGCAAACTGACGTGGACAGCGTA
CCACAGATAAGTATTGAAGATATTTATTGTAAAATGTGCGACCTTAATTTCGATGGGGAA
CTTCTGCTAGAATATCGAAAGCTCTACGCAGCTTTTGACGATTTTCCTCCTCCTCGCTGA
GTGGCATCCCTGGGTACAAGCGCACTTCTGCAAACCCGGCCGAAGATAGCAGTGCTGCGG
TTTCGTCACTCTCACAGTATGTTTCTGGAATAAAGCGTTTTAAAACAGCTTCCGAAGTTT
TGTGATCATTACCGAATAGAGCCTTG *3'* (Isolate no.2)

*5'*GAAGGCGGGTATGTAATGTACATTCCATTTACTGGAAATGCCGTACATCTACACTATCGT
CTCATCGACTGCCTTAAATCTGCTTGCCGGGGATACCGTCTAATGGCTCATGTTTGGCAT
TCTACATTCGTACTTGTCGTGAGGCGCGACCGCGAACGGCAAACTGACGTGGACAGCGTA
CCACAGATAAGTATTGAAGATATTTATTGTAAAATGTGCGACCTTAATTTCGATGGGGAA
CTTCTGCTAGAATATCGAAAGCTCTACGCAGCTTTTGACGATTTTCCTCCTCCTCGCTGA
GTGGCATCCCTGGGTACAAGCGCACTTCTGCAAACCCGGCCGAAGATAGCAGTGCTGCGG
TTTCGTCACTCTCACAGTATGTTTCTGGAATAAAGCGTTTTAAAACAGCTTCCGAAGTTT
TGTGATCATTACCGAATAGAGCCTTG *3'* (Isolate no. 3)

Phylogenetic study was performed for determining the genetic relationship of the DPV isolates with DPV isolates available in the GenBank. The evolutionary relationship was supposed using the Neighbour-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered along with the bootstrap test is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch length in the same units as those of the evolutionary distances used to infer the phylogenic tree. The evolutionary distances were computed using the highest Composite Likelihood Method (Tamura *et al.,* 2004). The tree derived from the sequence data is shown in Figure 17 and the estimates of evolutionary divergence between sequences is shown in Table 8.

Phylogenetic tree showed that sequenced strain of three DPVs of this study are (Anatid herpes 1 BAU DP1, DP2 and DP3) were similar with Anatid herpes virus 1 isolates originated from Vietnam (LC105645.1), China (EF643559.1), India (KJ958921.1, KM012009.1 and KX511893.1) and Bangladesh (KX768734.1) (Figure 17). Phylogenetic study on Bangladeshi DPV isolates conducted by Ahamed *et al.* (2015) found genetic similarity with the DPV isolates originated from China.

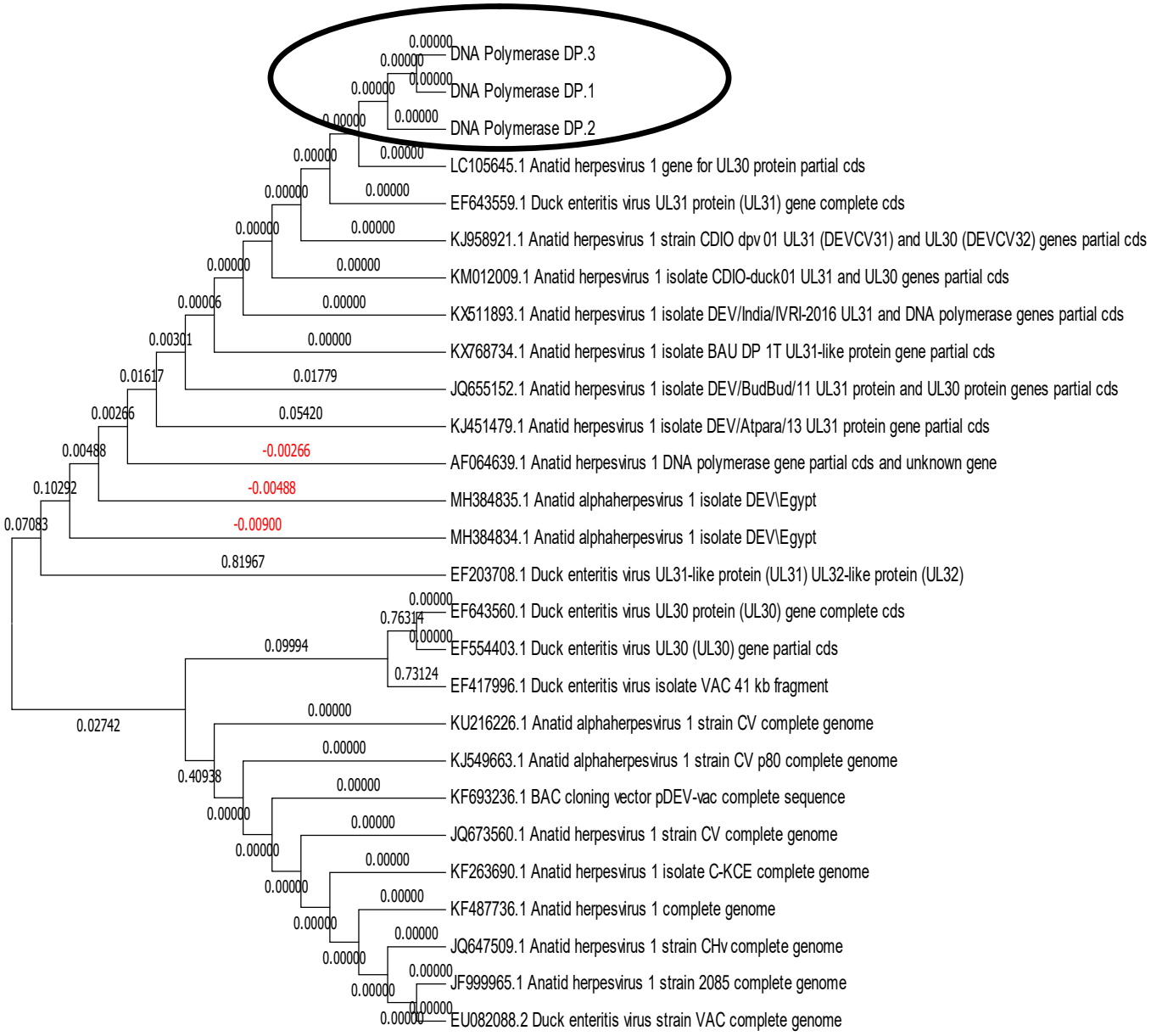

Figure 17: Phylogenetic relationship prepared from aligned sequences of the partial (446-bp) DNA polymerase gene of duck plague virus. The round box indicates the three Bangladeshi DPV isolates (DP.1, DP.2 and DP.3) of this study at the top.

Table 8. Estimates of evolutionary divergence between sequences

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
[1]																										
[2]	0.629																									
[3]	0.000	0.629																								
[4]	0.000	0.629	0.000																							
[5]	0.000	0.629	0.000	0.000																						
[6]	0.000	0.629	0.000	0.000	0.000																					
[7]	0.000	0.629	0.000	0.000	0.000	0.000																				
[8]	0.000	0.629	0.000	0.000	0.000	0.000	0.000																			
[9]	0.000	0.629	0.000	0.000	0.000	0.000	0.000	0.000																		
[10]	0.000	0.629	0.000	0.000	0.000	0.000	0.000	0.000	0.000																	
[11]	1.128	1.072	1.128	1.128	1.128	1.128	1.128	1.128	1.128	1.128																
[12]	0.629	0.000	0.629	0.629	0.629	0.629	0.629	0.629	0.629	0.629	1.072															
[13]	0.675	0.018	0.675	0.675	0.675	0.675	0.675	0.675	0.675	0.675	1.022	0.018														
[14]	0.597	0.018	0.597	0.597	0.597	0.597	0.597	0.597	0.597	0.597	1.040	0.018	0.036													
[15]	0.597	0.018	0.597	0.597	0.597	0.597	0.597	0.597	0.597	0.597	1.040	0.018	0.036	0.000												
[16]	0.597	0.018	0.597	0.597	0.597	0.597	0.597	0.597	0.597	0.597	1.040	0.018	0.036	0.000	0.000											
[17]	0.629	0.000	0.629	0.629	0.629	0.629	0.629	0.629	0.629	0.629	1.072	0.000	0.018	0.018	0.018	0.018										
[18]	0.719	0.057	0.719	0.719	0.719	0.719	0.719	0.719	0.719	0.719	1.214	0.057	0.078	0.057	0.057	0.057	0.057									
[19]	0.629	0.000	0.629	0.629	0.629	0.629	0.629	0.629	0.629	0.629	1.072	0.000	0.018	0.018	0.018	0.018	0.000	0.057								
[20]	0.629	0.000	0.629	0.629	0.629	0.629	0.629	0.629	0.629	0.629	1.072	0.000	0.018	0.018	0.018	0.018	0.000	0.057	0.000							
[21]	1.387	0.949	1.387	1.387	1.387	1.387	1.387	1.387	1.387	1.387	1.671	0.949	0.899	0.915	0.915	0.915	0.949	1.092	0.949	0.949						
[22]	1.385	1.078	1.385	1.385	1.385	1.385	1.385	1.385	1.385	1.385	1.494	1.078	1.132	1.041	1.041	1.041	1.078	1.231	1.078	1.078	1.739					
[23]	1.385	1.078	1.385	1.385	1.385	1.385	1.385	1.385	1.385	1.385	1.494	1.078	1.132	1.041	1.041	1.041	1.078	1.231	1.078	1.078	1.739	0.000				
[24]	0.629	0.000	0.629	0.629	0.629	0.629	0.629	0.629	0.629	0.629	1.072	0.000	0.018	0.018	0.018	0.018	0.000	0.057	0.000	0.000	0.949	1.078	1.078			
[25]	0.629	0.000	0.629	0.629	0.629	0.629	0.629	0.629	0.629	0.629	1.072	0.000	0.018	0.018	0.018	0.018	0.000	0.057	0.000	0.000	0.949	1.078	1.078	0.000		
[26]	0.629	0.000	0.629	0.629	0.629	0.629	0.629	0.629	0.629	0.629	1.072	0.000	0.018	0.018	0.018	0.018	0.000	0.057	0.000	0.000	0.949	1.078	1.078	0.000	0.000	
[27]	0.629	0.000	0.629	0.629	0.629	0.629	0.629	0.629	0.629	0.629	1.072	0.000	0.018	0.018	0.018	0.018	0.000	0.057	0.000	0.000	0.949	1.078	1.078	0.000	0.000	0.000

Legends= Bold highlighted numbers of antatid herpes virus showed no divergence with three DPV isolates of Bangladesh

[1] #KU216226.1_Anatid_alphaherpesvirus_1_strain_CV_complete_genome,
[2] #KX768734.1_Anatid_herpesvirus_1_isolate_BAU_DP_1T_UL31-like_protein_gene_partial_cds
[3] #KJ549663.1_Anatid_alphaherpesvirus_1_strain_CV_p80_complete_genome
[4] #KF693236.1_BAC_cloning_vector_pDEV-vac_complete_sequence
[5] #JQ673560.1_Anatid_herpesvirus_1_strain_CV_complete_genome
[6] #KF263690.1_Anatid_herpesvirus_1_isolate_C-KCE_complete_genome
[7] #KF487736.1_Anatid_herpesvirus_1_complete_genome
[8] #JQ647509.1_Anatid_herpesvirus_1_strain_CHv_complete_genome
[9] #JF999965.1_Anatid_herpesvirus_1_strain_2085_complete_genome
[10] #EU082088.2_Duck_enteritis_virus_strain_VAC_complete_genome
[11] #EF417996.1_Duck_enteritis_virus_isolate_VAC_41_kb_fragment
[**12] #KX511893.1_Anatid_herpesvirus_1_isolate_DEV/India/IVRI-**
2016_UL31_and_DNA_polymerase_genes_partial_cds
[13] #JQ655152.1_Anatid_herpesvirus_1_isolate_DEV/BudBud/11_UL31_protein_and_UL30_protein_ genes_partial_cds
[14] #AF064639.1_Anatid_herpesvirus_1_DNA_polymerase_gene_partial_cds_and_unknown_gene
[15] #MH384835.1_Anatid_alphaherpesvirus_1_isolate_DEV\Egypt
[16] #MH384834.1_Anatid_alphaherpesvirus_1_isolate_DEV\Egypt
[17] #KM012009.1_Anatid_herpesvirus_1_isolate_CDIO-duck01_UL31_and_UL30_genes_partial_cds
[18] #KJ451479.1_Anatid_herpesvirus_1_isolate_DEV/Atpara/13_UL31_protein_gene_partial_cds

[19] **#KJ958921.1_Anatid_herpesvirus_1_strain_CDIO_dpv_01_UL31_(DEVCV31)_and_UL30_(DEVCV32)_genes_partial_cds**
[20] #EF643559.1_Duck_enteritis_virus_UL31_protein_(UL31)_gene_complete_cds
[21] #EF203708.1_Duck_enteritis_virus_UL31-like_protein_(UL31)_UL32-like_protein_(UL32)
[22] #EF643560.1_Duck_enteritis_virus_UL30_protein_(UL30)_gene_complete_cds
[23] #EF554403.1_Duck_enteritis_virus_UL30_(UL30)_gene_partial_cds
[24] #LC105645.1_Anatid_herpesvirus_1_gene_for_UL30_protein_partial_cds
[25] #DNA_Polymerase_DP.2
[26] #DNA_Polymerase_DP.3
[27] #DNA_Polymerase_DP.1

12. Research highlight/findings:

- A total of 10 DPV isolates (04 from Mohanganj, 01 from Mymensingh, 03 from Nilphamari and 02 from Safari Park, Gazipur) from 18 samples in 04 field outbreaks were identified by PCR of the virus DNA.
- Eight duck plague virus isolates were adapted in the embryonated duck eggs.
- Molecular characterization by phylogenetic analysis of the DPV isolates showed 100% sequence similarity with DPV isolates originated from Bangladesh, Vietnam and China.

B. Implementation Position

1. Procurement:

Description of equipment and capital items	PP Target		Achievement		Remarks
	Phy (#)	Fin (Tk)	Phy (#)	Fin (Tk)	
(a) Office equipment	*GD4 & GD6	101000.00	*GD4 & GD6	100700.00	Tk. 300/-could not spend
(b) Lab &field equipment	*GD1 & GD3	584850.00	*GD1 & GD3	584400.00	Tk. 450/- could not spend
(c) Other capital items					

*= Procurement package no

Procurement (List of items)

Package No.	Description of Procurement Package Goods	Unit	Quantity
1	2	3	4
GD_1	**Procurement of Equipment:**		
	a) -20 ºC medical refrigerator (12 CFT)	No.	01
	b) Micropipette sets		
	i) Single channel: (0.5-10 µl, 10-100µl, 50-300µl, 200-1000µl)	No.	04
	ii) Multichannel (For ELISA) 50-300µl	No.	01
	c) i. Computer,	No.	01
	ii. printer	No.	01
	iii.UPS etc.	No.	01
	d) Ice Box	No.	01
	e) Sample storage box	No.	05
	f) Micropipette holding rack	No.	01
	g) Scanner	No.	01
	h) Egg incubator	No.	01
GD_3	**Procurement of chemicals for molecular research:**		
	a) Genomic DNA extraction kit (100 reaction/kit)	No.	03
	b) 2×PCR master mix (100 reaction/kit)	No.	10
	c) PCR primers	No.	06
	d) DNA purification kit	No.	02
	e) Agarose (100g/pack) (electrophoresis grade)	No.	03
	f) Agar Nobel (For AGIDT) (100g/pack)	No.	01
	g) PCR tubes 0.2ml (500tubes/pack)	No.	02
	h) DNA sequencing	No.	
GD_4	**Procurement of Chemicals for Biochemical test and Serogrouping**		
	a) Cultural media (Blood agar base) (500g/pack)	No.	02
	b) Duck plague virus refernce antiserum	No.	01
	c) Sodium Azide (For AGIDT)	No.	01
	d) 100 bp DNA ladder-5	No.	05
	e) Disposables plastic pipettes of different sizes:10 ml, 5ml, 2 ml, 1 ml-	No.	10

Package No.	Description of Procurement Package Goods	Unit	Quantity
1	2	3	4
	10 f) Disposables tips for Micropipettes of different grades-20 g) Antibiotic and antifungal , (Gentamycin and nystatin) h) Ethidium bromide i) PBS (Sigma) j) NaCl (Sigma) k) Ethanol absolute l) Glycerol m) Phenol n) Spirit o) EDTA p) Tris-Hcl q) Eppendrof tube (1000pc) r) Glass bottle (1L) s) Conical flask (500 ml) t) Test tube u) Filter (0.2 mµ) v) ELISA kit w) Disposable plastic syringe(100 pack)	No. No.	20 02 05 01 02 01 02 01 01 01 03 05 04 100 01 02 10
GD_6	**Procurement of the Furniture:** **a)** File cabinet **b)** Visitors chair **c)** Computer table	No. No. No.	01 03 01

2. Establishment/renovation facilities:

Description of facilities	Newly established		Upgraded/refurbished		Remarks
	PP Target	Achievement	PP Target	Achievement	
Repair , renovation and maintenance of duck shed, pond with fencing			100000.00	100000.00	

3. Training/study tour/ seminar/workshop/conference organized: Not applicable

Description	Number of participant			Duration (Days/weeks/ months)	Remarks
	Male	Female	Total		
(a) Training					
(b) Workshop					

C. Financial and physical progress

Fig in Tk

Items of expenditure/activities	Total approved budget	Fund received	Actual expenditure	Balance/ unspent	Physical progress (%)	Reasons for deviation
A. Contractual staff salary	317150	310140	309890	250	97.8%	
B. Field research/lab expenses and supplies	1077000	1077000	1076340	660	99.9%	
C. Operating expenses	170000	163173	158279	4894	92.5%	
D. Vehicle hire and fuel, oil & maintenance	50000	50000	50000	0	100%	
E. Training/workshop/seminar etc.	0	0	0	0		
F. Publications and printing	75000	0	0	75000	0%	fund not released
G. Miscellaneous	25000	25000	25000	0	100%	
H. Capital expenses	685850	685850	685100	750	99.9%	

D. Achievement of Sub-project by objectives: (Tangible form)

Specific objectives of the sub-project	**Major technical activities performed in respect of the set objectives**	**Output (i.e. product obtained, visible, measurable)**	**Outcome(short term effect of the research)**
Isolation, identification and characterization of duck plague virus (DPV) from field outbreaks	• Collection of samples from field outbreaks • Isolation of virus using embryonated eggs of duck • Molecular detection of DPV by PCR • Molecular characterization by sequencing and phylogenetic analysis	Fully characterized (molecular characterization at the level of phylogenetic analysis of the isolated DVA) virulent DPV virus isolates available at the laboratory repository.	Isolated DPV could be used as seed virus for vaccine production
Development of live attenuated duck plague vaccine from local field strains	Attenuation of DPV by serial passage into the embryonated chicken eggs	Attenuation of duck plague virus up to 10 passages has been completed. A total of 46 passages is need for complete attenuation of a virulent DPV.	-
Determination of efficacy of the developed vaccine	No technical activity could be started since attenuation has not been completed due to time constraints	-	-

E. Materials Development/Publication made under the Sub-project:

Publication	Number of publication		Remarks (e.g. paper title, name of journal, conference name, etc.)
	Under preparation	Completed and published	
Technology bulletin/ booklet/leaflet/flyer etc.	-	-	-
Journal publication	-	-	-
Information development	-	-	Results of sequencing analysis but not published
Other publications, if any		MS thesis	Characterization of Duck Plague Virus Isolated from Field outbreaks

F. Technology/Knowledge generation/Policy Support (as applied):

i. Generation of technology (Commodity & Non-commodity)

Local strain of duck plague virus isolated and characterized at the molecular level with phylogenetic analysis.

ii. **Generation of new knowledge that help in developing more technology in future**

The knowledge generated through this project related to isolation, identification, characterization and attenuation techniques of duck plague virus could be used for future live vaccine production.

iii Technology transferred that help increased agricultural productivity and farmers' income

None

iii. Policy Support

None

G. Information regarding Desk and Field Monitoring

i) Desk Monitoring [description & output of consultation meeting, monitoring workshops/seminars etc.): Not applicable

ii) Field Monitoring (time& No. of visit, Team visit and output):

Time	No. of visit	Team visit	Output
04/03/2018	01	Technical Division/ Unit, BARC	Satisfactory
07/03/2018	01	PIU-BARC, NATP-2	Satisfactory

H. Lesson Learned (if any)

- For successful completion of this type of vaccine development project needed more time.

I. Challenges (if any)

- Duck plague outbreaks is seasonal that started from February. Consequently implementation of the project activities related to virus isolation, identification and characterization was delayed.
- Attenuation of duck plague virus for live vaccine development using chicken embryos requires 41-46 passages that needs at least one year.
- Keeping pace with the time was challenging.

Signature of the Principal Investigator
Date …….
Seal

Counter signature of the Head of the organization/authorized representative
Date ……..
Seal

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